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Original Article

Hepatitis B virus genotypes, precore mutations, and basal core promoter mutations in HBV-infected Chinese patients with persistently normal alanine aminotransferase and low serum HBV-DNA levels

Ming Shi[a*], *Yong Zhang*[a], *Jing Zhang*[b], *Wei Liu*[a], *Lu Xing*[a]

[a]*Central Laboratory, No.6 Hospital of Dalian, Liaoning Province, China*
[b]*Department of Clinical Laboratory, Dalian Central Hospital, Liaoning Province, China*

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ABSTRACT

Hepatitis B virus (HBV) genotype and precore and basal core promoter (BCP) mutants in the patients with persistently normal alanine aminotransferase (ALT) and low serum HBV-DNA levels are unclear. The aim of this study was to determine HBV genotypes, precore and BCP mutations, and their association with chronic hepatitis and liver fibrosis in HBV-infected patients with persistently normal ALT, and low serum HBV-DNA levels in northeast China. Patients (n = 89) with normal ALT and serum HBV-DNA levels below 20000 IU/mL but detectable with real-time PCR were included in this study. HBV genotypes were determined by real-time PCR. The precore and BCP mutations were detected by sequencing. All the patients had biopsy results. Of the 89 patients, 11 (12.4%) were genotype B and 78 (87.6%) were genotype C. The most common mutations were G1896A (23.6%), G1764A (9.0%), and A1762T (6.7%). The prevalence of precore mutation was significantly higher in genotype B patients than in genotype C patients (54.5% vs. 19.2%, $p < 0.01$). There was no significant difference in the prevalence of BCP mutations between genotype B and genotype C (18.2% vs. 10.2%). Multivariate analysis showed that old age (> 40 years) and BCP mutations were independent predictors of liver necroinflammation and fibrosis. Thus, BCP mutations may be associated with liver necroinflammation and fibrosis in patients with persistently normal ALT and low serum HBV-DNA levels in northeast China.

Introduction

Hepatitis B virus (HBV) infection is a public health problem worldwide. It is estimated that approximately 3 billion people have been exposed to HBV, of whom more than 350 million are chronically infected.[1] Based on an intergroup divergence of 8% or more in the full-length nucleotide sequence, HBV has been classified into 8 major genotypes (designated from A to H), and most of them show a distinct geographic distribution.[2,3] Genotypes B and C are the predominant HBV genotypes in Eastern Asia, including China.[4] There is growing evidence that HBV genotypes are associated with severity of liver diseases, certain prognoses, and response to antiviral therapies.[4-7]

* *Corresponding author at:* 269 Guibai Road, Dalian 116031, Liaoning Province, China
E-mail address: zhsh6655@sohu.com (Ming Shi)

In addition to natural polymorphic variances, HBV can evolve certain variants in the precore and basal core promoter (BCP) regions.[8,9] The G to A substitution at nucleotide 1896 (G1896A) is one of the most common mutations in the precore region, which prevents the production of hepatitis B e antigen (HBeAg) by introducing a premature stop codon into the open reading frame of the precore region. Thus, this mutation is frequently detected in patients with HBeAg-negative chronic hepatitis B.[10] Another common mutation is a double mutation in BCP region involving an A to T substitution at nucleotide 1762 and a G to A substitution at nucleotide 1764 (A1762T/G1764A), which aborts the transcription of precore mRNA but does not seriously affect that of viral pregenome RNA.[11] Although the frequencies of the precore and BCP mutations vary between HBV genotypes, they are associated with fulminant hepatitis B and hepatocellular carcinoma (HCC).[12,13]

The level of circulating viraemia is a risk factor for the development of cirrhosis and HCC, and is used to diagnose hepatitis B infection and define the response to antiviral therapy.[14,15] Since chronic hepatitis, cirrhosis and HCC have been found in patients with low serum HBV DNA-levels, the current cutoff value of 20,000 IU/mL is still debatable. Further understanding the virological characteristics in patients with low HBV-DNA levels will be helpful for clinical decisions in management of patients with low circulating viraemia. Many studies have demonstrated that HBV genotype and precore and BCP mutants have a substantial impact on the progression of chronic hepatitis B. However, few of them have been performed in patients with persistently normal alanine aminotransferase (ALT) and low serum HBV DNA levels.[4,16,17] In the present study, we aimed to examine the prevalence of HBV genotypes and G1896A mutation and A1762T/G1764A double mutations and their association with status of chronic hepatitis B in HBV-infected patients with persistently normal ALT and low serum HBV-DNA levels in Liaoning, a northeast province of China.

Material and methods

Study population and samples

From May 2003 to December 2009, 89 patients were selected from the patients attending Dalian Central Hospital and Dalian No. 6 Hospital (a hospital for infectious diseases). Patients were all HBsAg positive for at least six months and had detectable serum HBV-DNA but lower than 20,000 IU/mL by real-time PCR. All the patients were monitored for ALT levels every month, and the results were consistently normal (lower than 30 IU/L) for at least six months before enrollment. Patients were excluded from the study if they had any of the following conditions: 1) previous treatment for HBV infection, 2) co-infection with hepatitis A, C, D and E virus or HIV, 3) history of alcohol or drug abuse, and 4) other possible causes of chronic liver damage. Serum samples were collected from these patients and stored at -70ºC until use. The study protocol conformed to the Declaration of Helsinki, and was approved by the Ethics Committees of the institutions. Every patient gave his/her informed consent for this study.

Detection of ALT, HBV DNA, and the serum markers of HBV infection

Hepatitis B surface antigen (HBsAg), hepatitis B surface antibody (HBsAb), HBeAg, anti-HBe, and hepatitis B core antibody (anti-HBc) were measured with commercially available reagents (Kehua Biotech, Shanghai, China) according to manufacturer's instruction. ALT activity was determined kinetically on Hitachi 7600 automatic biochemistry analyzer with commercial kits (Roche Diagnostics – Penzberg, Germany). HBV-DNA levels were tested using real-time PCR kits (Fosun Diagnostics – Shanghai, China) on ABI Prism 7500 PCR System (Applied Biosystems Inc – Foster City, CA, USA). The lower detection limit of this kit is 100 IU/mL (500 copies/mL).[18] All reagents were approved by Chinese State Food and Drug Administration for in vitro diagnostic use.

Detection of G1896A precore mutation and A1762T/G1764A double mutations

Precore 1896 and BCP 1762/1764 mutations were assayed using direct DNA sequence analysis as described by Yang et al.[13] Briefly, HBV-DNA was isolated from 100 µL of serum sample with a QIAamp DNA Mini Kit (Qiagen – Hilden, Germany) according to the manufacturer's instruction. HBV-DNA was amplified with nested PCR on an ABI 9600 thermocycler (Applied Biosystem – Foster City, CA, USA). Final PCR products were checked by agarose gel electrophoresis and purified before sequence analysis using ABI PRISM BigDye and ABI 3730 sequencer (Applied Biosystem – Foster City, CA, USA), according to the manufacturer's instruction.

Genotyping of HBV

HBV genotypes were determined by a commercial real-time PCR kit (Fosun Diagnostics – Shanghai, China). This kit was approved by Chinese State Food and Drug Administration for in vitro diagnosis use. The kit only detects HBV genotype B and C with a lower detection limit of 200 IU/mL. Samples that could not be detected by the real time PCR kit were subjected to DNA sequence analysis of the pre-S region of the HBV genome as described by Yeh et al.[19]

Histological assessment of the liver

The degree of hepatic inflammation and stage of fibrosis was determined with the use of Batts-Ludwig's grading and staging system for chronic hepatitis.[20] All the 89 patients had liver biopsy results.

Statistical analysis

Statistical analyses were performed with the Statistical Program for Social Sciences (SPSS 13.0 for Windows, SPSS, Chicago, IL, USA). Qualitative and quantitative data were compared by chi-square test, Fisher's exact test, and Mann-Whitney test, when applicable. The association between severity of histology and mutations was determined by multiple logistic regression. Statistical significance was denoted as a p-value less than 0.05.

Results

Demographic and clinical characteristics of the patients

The study population consisted of 89 patients with low serum HBV DNA loads (detectable serum HBV-DNA but lower than 20000 IU/mL). Twenty-nine of them were HBeAg negative and 60 were HBeAg positive. Liver biopsy showed chronic active hepatitis and cirrhosis in eight patients (9.0%). All the 60 HBeAg positive patients had HBV-DNA less than 20000 IU/mL, ALT lower than 30 IU/L, and normal biopsy results. Twenty-one of the HBeAg negative patients were inactive HBV carriers (HBV-DNA less than 2000 IU/mL, ALT lower than 30 IU/L, and normal biopsy results). The demographic and clinical characteristics were shown in Table 1.

Distribution of HBV genotypes

Of the 89 patients, 11 (12.4%) were genotype B and 78 (87.6%) were genotype C. The B to C ratio showed significant differences between HBeAg positive and HBeAg negative patients ($p < 0.05$). Compared to patients with genotype C, patients with genotype B had lower viral load, higher HBeAg negative rate, and older (Table 2).

Table 1 - Demographic, biochemical, and virological characteristics of the studied patients

	HBeAg-positive (n = 60)	HBeAg-negative (n = 29)
Age (years) Mean ± SD	35.6 ± 6.8	47.2 ± 8.1
Men/Women	39/21	21/8
ALT (IU/L) Mean ± SD	22.3 ± 7.3	24.2 ± 5.7
HBV-DNA (log_{10} IU/mL) Mean ± SD	3.43 ± 0.81	3.15 ± 0.32
Necroinflammation	0	8
Fibrosis	0	8

Table 2 - Frequency of precore and basal core promoter mutations stratified by HBeAg, genotype, and histology

	HBeAg-positive (n = 60)	HBeAg-negative (n = 29)	p
HBeAg- (%)	8 (72.8)	21 (26.9)	< 0.01
HBV-DNA (log_{10} IU/mL) Mean ± SD	2.34 ± 0.58	3.76 ± 0.63	< 0.01
Age (Mean ± SD)	48 ± 5.5	39 ± 7.9	< 0.01
BCP mutation	2 (18.2)	8 (10.2)	> 0.05
A1762T	1 (9.1)	1 (1.3)	-
G1764A	1 (9.1)	3 (3.8)	-
A1762T/G1764A	0	4 (5.1)	-
Precore mutation	6 (54.5)	15 (19.2)	< 0.01
G1896A	6 (54.5)	15 (19.2)	< 0.01

HBV genotypes and precore and BCP mutations

The precore and BCP mutations occurred in 21 (23.6%) and 10 (11.2%) patients, respectively (Table 2). The most common mutations were G1896A, G1764A, and A1762T [21 (23.6%), 8 (9.0%), and 6 (6.7%), respectively]. Two patients (2.2%) had mixed mutations of G1896A and G1764A. Four (4.5%) patients had mixed mutations of A1762T/G1764A. One patient (1.1%) had G1764A/C1766G mutation. The prevalence of precore mutation was significantly higher in genotype B patients (54.5%) than in genotype C patients (19.2%, $p < 0.01$). However, there was no significant difference in the prevalence of BCP mutations between genotype B and genotype C patients. The precore and BCP mutations occurred more frequently in patients older than 40 years ($p < 0.05$).

All the 29 HBeAg negative patients had precore and/or BCP mutations [19 (65.5%) with precore mutation, 8 (27.6%) with BCP mutation, and 2 (6.9%) with mixed mutations of precore and BCP]. There were no significant differences in HBV-DNA levels between HBeAg positive and negative patients. Due to small number of genotype B patients in our study, we evaluated the relationship among HBV-DNA levels and precore and BCP mutations among patients with genotype C. HBV-DNA levels were significantly lower in patients with the precore mutation than in those without the mutation ($p < 0.01$). However, HBV-DNA levels were significantly higher in patients with BCP mutations than in those without the mutations ($p < 0.01$).

HBV genotypes, precore and BCP mutations, and histological assessment

There were 8 patients (9.0%) with necroinflammatory activity (G1-G2) and fibrosis (S1-S2) simultaneously. All the 8 patients were genotype C and had BCP mutations (three of them had mixed mutations of precore and BCP). Although two patients had BCP mutations among patients with genotype B, they had no liver inflammation or fibrosis. Logistic regression with the variables age (> 40 years), genotype, precore and BCP mutations, and HBV-DNA level (> 2000 IU/mL) showed that age and BCP mutations were independent predictors of liver necroinflammation and fibrosis.

Discussion

Although treatment criteria for patients with HBV infection are well established, the optimal management of patients with persistently normal ALT levels and low serum HBV-DNA levels has always been debatable. It is now recognized that lower serum HBV-DNA levels may be associated with progressive liver disease and may warrant treatment, particularly in those who are HBeAg negative or have already developed cirrhosis.[14] Currently, the widely accepted cut-off value of serum HBV-DNA is 20000 IU/mL (100000 copies/mL) for HBeAg positive patients and 2000 IU/mL for HBeAg negative patients, respectively. For patients with increased ALT and HBV-DNA above 20000 IU/mL, biopsy does not usually affect the decision for treatment.[21] For patients with normal ALT and HBV-DNA < 20000 IU/mL, there are no any agreed diagnostic and

treatment criteria. The virological characteristics in these patients also remain unclear. In the present study, we selected 89 Chinese patients with persistently normal ALT and serum HBV-DNA < 20000 IU/mL but detectable with real-time PCR, to examine the prevalence of HBV genotypes, the precore mutation, BCP mutations, and their association with status of chronic hepatitis B. HBV genotype C (87.6%) was dominant in these patients. The precore and BCP mutations were observed in 23.6% and 11.2% of the patients, respectively. All the 29 HBeAg negative patients had precore or BCP mutations. Eight patients (9.0%) with genotype C, HBeAg negative, and BCP mutations were found with liver necroinflammation and fibrosis. These results imply that detection of BCP mutations may be a useful tool in management of patients with persistently normal ALT and low HBV-DNA levels in addition to liver biopsy.

Previous studies show that the prevalence of precore and BCP mutations depends on HBV genotypes, and these mutations are related to fulminant and severe hepatitis and hepatocellular carcinoma.[4,5,22-25] Some studies have reported that genotype C, BCP mutations, old age, high ALT levels and low albumin levels are associated with higher degree of necroinflammation as well as fibrosis by univariate analyses, and BCP mutations are associated with genotype C.[26-28] However, only high ALT levels were independently correlated with high histological activity index (HAI) scores.[26] Our results show that the prevalence of BCP mutations has no significant differences between genotypes B and C. The discrepancy between our results and previous studies may be due to the small number of genotype B patients in our study and the special characteristics of our subjects. Our results also show that BCP mutations are associated with higher HBV-DNA levels in genotype C patients, and this is consistent with previous studies.[29,30]

Eight patients (9.0%) had liver necroinflammation and fibrosis in our study. All these patients were genotype C, HBeAg negative, older, and with BCP mutations. Two patients with BCP mutation and genotype B had normal liver histology. Yuen et al.[26] reported that BCP mutations were associated with more severe necroinflammation but not with the degree of fibrosis. The eight patients in our study had necroinflammation and fibrosis simultaneously. However, the findings in our study were not sufficient to support the conclusion that the effects of BCP mutations exerted on necroinflammation might lead to liver fibrosis. Further studies are needed to clarify the effect of BCP mutations on the progression of chronic hepatitis B.

In conclusion, necroinflammation and fibrosis exist in Chinese patients with persistently normal ALT and low serum HBV-DNA levels. BCP mutations may be associated with liver necroinflammation and fibrosis in these patients. Further studies are needed to confirm the significance of such association.

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Conflict of interest

All authors declare to have no conflict of interest.

REFERENCES

1. Lee WM. Hepatitis B virus infection. N Engl J Med. 1997;337:1733-45.
2. Okamoto H, Tsuda F, Sakugawa H, et al. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. J Gen Virol. 1988;69:2575-83.
3. Norder H, Courouce AM, Coursaget P, et al. Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. Intervirology. 2004;47:289-309.
4. Kao JH, Chen PJ, Lai MY, et al. Hepatitis B genotypes correlate with clinical outcomes in patients with chronic hepatitis B. Gastroenterology. 2000;118:554-9.
5. Sanchez-Tapias JM, Costa J, Mas A, et al. Influence of hepatitis B virus genotypes on the long term outcome of chronic hepatitis B in Western patients. Gastroenterology. 2002;123:1848-56.
6. Yuen MF, Sablon E, Wong DK, et al. Role of hepatitis B virus genotypes in chronic hepatitis B exacerbation. Clin Infect Dis. 2003;37:593-7.
7. Kao JH, Wu NH, Chen PJ, et al. Hepatitis B genotypes and the response to interferon therapy. J Hepatol. 2000;33:998-1002.
8. Bartholomeusz A, Locarnini S. Hepatitis B virus mutants and fulminant hepatitis B: fitness plus phenotype. Hepatology. 2001;34:432-5.
9. Liang TJ, Hasegawa K, Rimon N, et al. A hepatitis B virus mutant associated with an epidemic of fulminant hepatitis. N Engl J Med. 1991;324:1705-9.
10. Funk ML, Rosenberg DM, Look AS. World-wide epidemiology of HBeAg-negative chronic hepatitis B and associated precore and core promoter variants. J Viral Hepat. 2002;9:52-61.
11. Okamoto H, Tsuda F, Akahane Y, et al. Hepatitis B virus with mutations in the core promoter for an e antigen-negative phenotype in carriers with antibody to e antigen. J Virol. 1994;68:8102-10.
12. Chan HL, Hussian M, Lok AS. Different hepatitis B virus genotypes are associated with different mutations in the core promoter and precore regions during hepatitis B e antigen seroconversion. Hepatology. 1999;29:976-84.
13. Yang HI, Yeh SH, Chen PJ, et al. Associations between hepatitis B virus genotype and mutants and the risk of hepatocellular carcinoma. J Natl Cancer Inst. 2008;100:1134-43.
14. Lok AS, McMahon BJ. Chronic hepatitis B: update 2009. Hepatology. 2009;50:1-36.
15. Alazawi W, Foster GR. Advances in the diagnosis and treatment of hepatitis B. Curr Opin Infect Dis. 2008; 21:508-15.
16. Chu CJ, Hussain M, Lok AS. Hepatitis B virus genotype B is associated with earlier HBeAg seroconversion compared with hepatitis B virus genotype C. Gastroenterology. 2002;122:1756-62.

17. Sumi H, Yokosuka O, Seki N, et al. Influence of hepatitis B virus genotypes on the progression of chronic type B liver disease. Hepatology. 2003;37:19-26.
18. Shi M, Zhang Y, Zhu YH, et al. Comparison of real-time polymerase chain reaction with the COBAS Amplicor test for quantitation of hepatitis B virus DNA in serum samples. World J Gastroenterol. 2008;14:479-83.
19. Yeh SH, Tsai CY, Kao JH, et al. Quantification and genotyping of hepatitis B virus in a single reaction by real-time PCR and melting curve analysis. J Hepatol. 2004;41:659-66.
20. Batts KP, Ludwig J. Chronic hepatitis. An update on terminology and reporting. Am J Surg Pathol. 1995;19:1409-17.
21. Papatheodoridis GV, Manesis EK, Manolakopoulos S, et al. Is there a meaningful serum HBV DNA cutoff level for therapeutic decisions in HBeAg-negative chronic hepatitis b virus infection? Hepatology. 2008;48:1451-9.
22. Omata M, Ehata T, Yokosuka O, et al. Mutations in the precore region of hepatitis B virus DNA in patients with fulminant and severe hepatitis. N Engl J Med. 1991;324:1699-704.
23. Kao JH, Chen PJ, Lai MY, et al. Basal promoter mutations of hepatitis B virus increase the risk of hepatocellular carcinoma in hepatitis B carriers. Gastroenterology. 2003;124:327-34.
24. Hayashi K, Katano Y, Takeda Y, et al. Association of hepatitis B virus subgenotypes and basal core promoter/precore region variants with the clinical features of patients with acute hepatitis. J Gastroenterol. 2008;43:558-64.
25. Victoria FD, Oliveira MC, Victoria MB, et al. Characterization of HBeAg-negative chronic hepatitis B in western Brazilian Amazonia. Braz J Infect Dis. 2008;12:27-37.
26. Yuen MF, Tanaka Y, Ng IO, et al. Hepatic necroinflammation and fibrosis in patients with genotypes Ba and C, core-promoter and precore mutations. J Viral Hepat. 2005;12:513-8
27. Orito E, Mizokami M, Sakugawa H, et al. A case-control study for clinical and molecular biological dirrerences between hepatitis B viruses of genotypes B and C. Japan HBV Genotype Research Group. Hepatolgy. 2001;33:218-23.
28. Yuen MF, Sablon E, Yuan HJ, et al. The relationship between the development of precore and core promoter mutations and HBeAg seroconversion in chronic hepatitis B. J Infect Dis. 2002;186:1335-8
29. Buchwold VE, Xu Z, Yen TS, et al. Effects of a frequent double-nucleotide basal core promoter mutation and its putative single-nucleotide precurcor mutations on hepatitis B virus gene expression and replication. J Gen Virol. 1997;78:2055-65.
30. Moriyama K, Okamoto H, Tusda F, et al. Reduced precore transcription and enhanced core-pregenome transcription of hepatitis B virus DNA after replacement of the precore-core promoter with sequences associated with e antigen-seronegative persistent infections. Virology. 1996;226:269-80.